Region-specific and dose-specific effects of chronic haloperidol exposure on [^{3}H]Flumazenil and [^{3}H]Ro15-4513 GABA$_A$ receptor binding sites in the rat brain

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Abstract

Data from *post-mortem* studies suggest that schizophrenia is associated with abnormal expression of $GABA_A$ receptor ($GABA_AR$) α subunits including $\alpha5GABA_A$. Positron emission tomography (PET) measures of $GABA_AR$ binding in schizophrenic patients, however, have not revealed consistent alterations *in vivo*. Animal studies using the $GABA_AR$ agonist [^{3}H]muscimol have provided evidence that antipsychotic drugs used in schizophrenia can influence $GABA_AR$ binding, in a region-specific manner, complicating the interpretation of the PET GABA signal in medicated patients. No binding data, however, are available for more subunit-selective ligands. To address this, we combined a rodent model of clinically relevant antipsychotic drug exposure with quantitative receptor autoradiography. Haloperidol (0.5 and 2 mg/kg/day) or vehicle were continuously administered to adult male Sprague-Dawley rats via osmotic pumps to maintain a clinically relevant, steady-state levels of drug exposure for 28 days. Quantitative receptor autoradiography was then performed *post-mortem* using the $GABA_A$ selective radioligand [^{3}H]Ro15-4513 and the non-subunit selective radioligand [^{3}H]flumazenil. Chronic haloperidol exposure increased [^{3}H]Ro15-4513 binding in the CA1 sub-field of the dorsal hippocampus ($p<0.01$; $q<0.01$). [^{3}H]flumazenil binding was also increased in most of the explored regions ($p<0.05$), independently of the dose of haloperidol used. This is the first study to demonstrate a region/dose-specific effect of haloperidol on [^{3}H]Ro15-4513 binding. Although caution needs to be exerted when extrapolating results from animals to patients, collectively these data confirm previous findings that antipsychotic treatment contributes to the heterogeneity observed in PET studies of $GABA_AR$ in schizophrenic patients, specifically at the $\alpha1/5GABA_AR$.

Introduction

γ-Amino-butyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system (CNS). The $GABA_A$ receptor ($GABA_AR$) is a pentameric GABA-gated chloride ion channel composed of several classes of subunits (α1–6, β1–3, γ1–3, δ, θ, ρ, and ε) [1]. Of these, the diversity of the α-subunit is thought to be responsible for shaping the functional properties and ligand selectivity of the $GABA_A$ benzodiazepine binding site ($GABA_A$-BZR) [2–5]. Benzodiazepines act at the α/γ interface for the α subunits 1, 3, 5 [6]. In the CNS, $GABA_A$-BZR mediate pyramidal cell activity via tonic and phasic inhibition [7–9].

Deficits in GABA neurotransmission, resulting in disruptions to normal patterns of neural oscillatory activity are implicated in the pathophysiology of schizophrenia [10–13]. In support of this, quantitative receptor autoradiography studies using [^{3}H]muscimol, an orthosteric agonist at the GABA binding site on $GABA_A$-BZR, provide consistent evidence for increased binding density in frontal and temporal cortices and the caudate nucleus in *post-mortem* brain tissue from patients with schizophrenia [14–19].

In contrast, *post-mortem* studies focusing specifically on mRNA expression of $GABA_A$ α-subunits report decreased expression of α1 [20,21], increased expression of α2 [21,22], but inconsistent results for the α5-subunit [21,23,24]. A systematic review of positron emission tomography (PET) studies in schizophrenia patients using selective radiotracers for the BZ-site of the $GABA_A$-BZR however found no consistent evidence for altered $GABA_A$-BZR availability in schizophrenia [25].

These *post-mortem* data come from patients with a long duration of illness and exposure to antipsychotic medication. Similarly in most of the PET studies, the patients were also receiving antipsychotic medication [25], and it has been shown that different

antipsychotics can directly alter the binding of ligands to $GABA_A$-BZR, presumably by altering the expression and availability of the receptors [26,27]. Hence, distinguishing effect(s) of illness from antipsychotic exposure is challenging and medication may represent a significant source of heterogeneity in these data.

In support of this view, pre-clinical studies in naïve adult rats show that chronic exposure to antipsychotic haloperidol directly influences binding of both [^{3}H]muscimol (indexing $GABA_AR$ binding) and [^{3}H]flunitrazepam (indexing BZ-site binding) in a duration of exposure and region-dependent manner [28–35]. These studies however have not been able to fully unravel this complex issue completely. While exposure of adolescent rats to antipsychotic drugs has recently been reported to increase [^{3}H]muscimol binding in the striatum and nucleus accumbens [36] a 12-day exposure to haloperidol is reported to decrease [^{3}H]flumazenil binding in several regions of the naïve rat brain [34]. Collectively, these data strongly suggest that exposure to antipsychotic medication influences $GABA_A$-BZR availability *in vivo*, but the direction of this effect remains ambiguous. Notably, these studies investigating effects of D_2 dopaminergic receptor (D2R)-based antipsychotics on $GABA_A$-BZR involved routes of administration that result in inappropriate pharmacokinetics that does not match a clinically comparable exposure [23].

The binding sites to the $GABA_A$-BZR allosteric ligand, [^{3}H]flumazenil, in the rodent and human brain have been shown to comprise both the "zolpidem-sensitive" and "zolpidem-insensitive sites", with the latter suggested to correspond to $GABA_ARs$ that contain the α5 subunit [38]. In a recent study, 12 days of systemic haloperidol exposure resulted in a significant reduction in zolpidem-sensitive binding sites (α1,2,3$GABA_AR$ [39]), with no effect on the insensitive-binding sites, suggesting a lack of effect on α5$GABA_ARs$

[34]. No studies however, have examined the potential impact of antipsychotic drug exposure using radioligands with greater selectivity for $GABA_A$-BZR containing α1/α5 subunits, such as Ro15-4513 [40,41]. This is relevant, since convergent lines of evidence from animal models strongly suggest that α5$GABA_AR$ have potential as a target for novel, non-dopaminergic antipsychotic compounds, by balancing hippocampal excitation via tonic inhibition of pyramidal neurons [42–49]. Notably, the efficacy of the compound SH-053-2'F-R-CH3, an *α*5$GABA_AR$ positive allosteric modulator (PAM), in the methylazoxymethanol acetate (MAM) neurodevelopmental disruption model of schizophrenia is compromised following prior exposure to the D2R antagonist haloperidol [41].

In the present study we therefore aimed to determine the impact of chronic exposure to haloperidol on $GABA_AR$ binding using *post-mortem* quantitative receptor autoradiography with [^{3}H]Ro15-4513 to assess *α*1/*α*5$GABA_AR$ and [^{3}H]flumazenil to assess BZ-sensitive *α*1-3;5$GABA_AR$ using a validated rat model of clinically comparable drug exposure [37,50]. Based on the results of McLeod and colleagues (2008) who observed decreases in zolpidem-sensitive binding sites and no change in zolpidem-insensitive sites after haloperidol exposure, we hypothesized that chronic haloperidol exposure would decrease [^{3}H]flumazenil binding, with no effect on [^{3}H]Ro15-4513 binding.

Methods

Animals and treatment protocol

Male Sprague-Dawley rats (N=36, Charles River, UK; ~ 10 weeks of age) were administered haloperidol (0.5 or 2 mg/kg/day; haloperidol; n=12/group: Sigma-Aldrich,

Gillingham, Dorset, UK) or vehicle (β-hydroxypropylcyclodextrin, 20% w/v, acidified to pH 6 using ascorbic acid; n=12/group) using osmotic minipumps for 28 days [31]. Dyskinetic behavior, i.e., vacuous chewing movements, was assessed once at 26 days after the start of haloperidol exposure. This involved a simple measurement of purposeless chewing jaw movements in a 2-minute period, outside the home cage as described previously [50]. All experimental procedures were performed in accordance with the relevant guidelines and regulations, specifically, the Home Office (Scientific Procedures) Act 1986, United Kingdom and European Union (EU) directive 2010/63/EU and the approval of the local Animal Welfare and Ethical Review Body (AWERB) panels at King's College London (for full details, see supplementary material).

Quantitative receptor autoradiography with [^{3}H]Ro15-4513 and [^{3}H]flumazenil

On completion of treatment, rats were terminally anesthetized and perfused. A plasma sample was collected for estimation of drug levels. Coronal 20 µm-thick sections were cut using a cryostat (Leica CM1950), mounted onto glass slides (Superfrost^{TM}) and stored at -80ºC until used for autoradiography (for further details, see supplementary material).

[^{3}H]Ro15-4513 (Perkin Elmer, NET925250UC) was used to quantify α1/5GABA$_A$R density. While this ligand has a high specificity (60-70%) [51] to α5GABA$_A$R, a smaller proportion of the binding has affinity to α1GABA$_A$R [52]. Non-specific binding was determined by bretazenil (Sigma, B6434-25MG) due to its affinity to bind to a variety of GABA$_A$R subtypes (α1-3;5) [53]. Sections were pre-incubated at room temperature in Tris buffer (50 mM) for 20 minutes followed by incubation in either 2 nM [^{3}H]Ro15-4513 for specific binding, or 2 nM of [^{3}H]Ro15-4513 and 10 µM of bretazenil for non-specific binding at room temperature for 60 minutes. Slides were then washed in Tris buffer (2 x 2 min) at room temperature, dipped in distilled water (dH$_2$0) and left to dry

overnight. Dry slides were placed into light-tight cassettes with a radioactive [^{3}H] standards slide, (ART-123A American Radiolabelled Chemicals Inc., USA) and hyperfilm (Amersham 8x10 in Hyperfilm Scientific Laboratory Supplies, UK). Films were exposed for 8 weeks before being developed in a Protex Ecomax film developer (Protec GmbH & Co, Germany). Identical procedures were used for [^{3}H]flumazenil (Perkin Elmer, NET757001MC), with the exception that the slides were incubated and washed in buffer at 4ºC with 1nM [^{3}H]flumazenil, and 10 µM flunitrazepam (Sigma Aldrich, F-907 1ML) and 1nM [^{3}H]flumazenil, for specific and non-specific binding, respectively, and exposed for 4 weeks before development.

Quantification of receptor binding

Films were developed and images were manually captured using a Nikon SLR camera and preprocessed (see supplementary material). Optical density (OD) measurements were obtained using MCID software (Imaging Research Inc., 2003) from *a priori* defined regions of interest (ROIs; Fig. 1). These were chosen based on the known distribution of α1/α5GABA$_A$R in the rat brain, data from prior studies reporting an effect of haloperidol on [^{3}H]muscimol or [^{3}H]flunitrazepam binding [31–34], and a defined role in the pathophysiology of schizophrenia [31,48,54–58]. Specific binding in nCi/mg was quantified using standard curves constructed from OD measurements of standards for each film, using the robust linear regression interpolation method in GraphPad (version 8.00, GraphPad Software, La Jolla California USA www.graphpad.com).

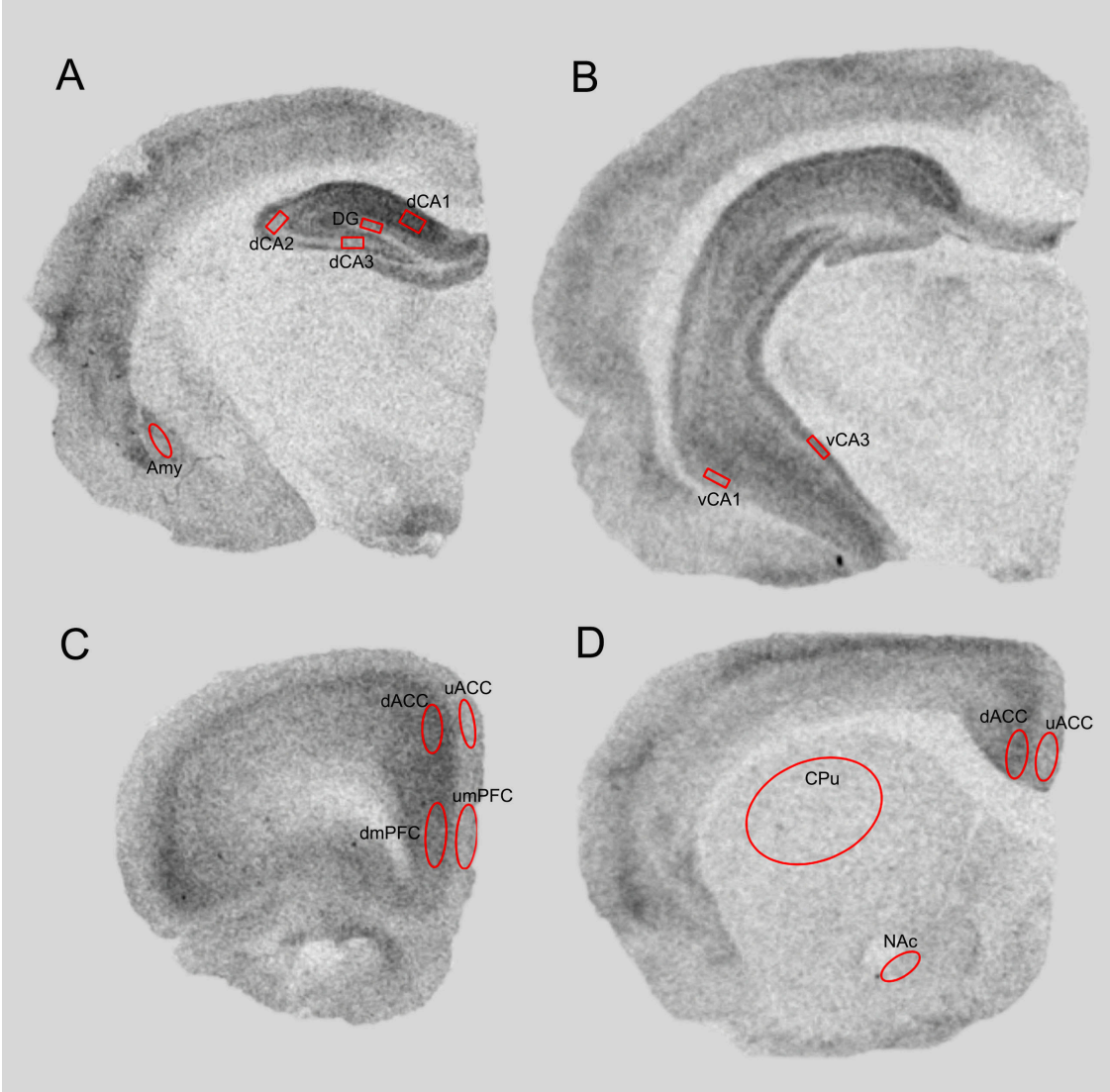

Figure 1. Representative [^{3}H]Ro15-4513 autoradiographs showing the placement of ROIs that were analyzed in this study. The same ROIs were used for the analysis of [^{3}H]flumazenil. A) dorsal hippocampal layers CA1 (dCA1), CA2 (dCA2), CA3 (dCA3), dentate gyrus (DG) and amygdala (amy). B) ventral hippocampal layers CA1 (vCA1), CA3 (vCA3). C) Medial Prefrontal Cortex (upper (1-3) and deeper (4-6) mPFC), Anterior Cingulate Cortex (upper (1-3) and deeper (4-6) ACC), differentiation of upper (1-3) vs deeper (4-6) layers was due to differential density of receptors across layers, specifically with α5 being more predominantly present in layer V and VI [59]. D) Caudate-Putamen (CPu), Nucleus Accumbens (NAc).

Statistical Analyses

All statistical analyses were performed in Prism software (v8.0.0 for Macintosh, GraphPad Software, La Jolla California USA, www.graphpad.com). The data were initially checked for significant outliers using Grubbs' test (α=0.05) with any significant outliers excluded from the final analysis. The data were then checked for Gaussian

distribution using the Shapiro-Wilk normality test. Autoradiographic data were normally distributed (Shapiro-Wilk, $p>0.05$), but vacuous chewing movements scores were not (Shapiro-Wilk, $p<0.01$). Group-level differences in ligand binding were assessed using mixed-effects model, with ROI as within-subject factor and treatment (vehicle, haloperidol 0.5 or 2 mg/kg/day) as between-subject factor, using the specific binding (nCi/mg) of either [^{3}H]Ro15-4513 or [^{3}H]flumazenil as the dependent variable. Vacuous chewing movements scores were analyzed using non-parametric Kruskal-Wallis test ($p<0.001$). *Post-hoc* tests were performed where appropriate and corrected for multiple comparisons using the 2-stage set-up method of Benjamini, Krieger and Yekutieli, with the false discovery rate set at 5% ($q<0.05$) [60]. Relationships between vacuous chewing movements and ligand binding were modeled using non-parametric Spearman's Rho correlation (2-tailed).

Results

Haloperidol plasma levels and vacuous chewing movement behavior

Administration of haloperidol by osmotic pump achieved plasma levels (mean ± s.d.) of 2.96 ± 0.52 ng/mL and 12.2 ± 1.96 ng/mL, for dose haloperidol 0.5 and 2 mg/kg/day, respectively. Stereotypical vacuous chewing movement behaviors were significantly different across treatment group (Kruskal-Wallis statistic = 9.98; $p<0.001$; Fig. S1). *Post-hoc* testing revealed a significant increase in vacuous chewing movements in those animals exposed to 2 mg/kg/day haloperidol after 26 days exposure, as compared to vehicle ($p<0.01$; $q<0.05$). There were no statistically significant differences between the haloperidol-exposed groups ($p>0.05$; $q>0.05$). Vacuous chewing movements were not related to the binding of either [^{3}H]Ro15-4513 (Table S1) or [^{3}H]flumazenil across any ROI (Table S2). Haloperidol plasma levels did not significantly correlate with binding of either of the ligands used (Table S3).

Dose-specific changes in [^{3}H]Ro15-4513 binding after haloperidol exposure measured with quantitative autoradiography

Mixed-effects model ANOVA revealed a statistically significant main effect of ROI ($F(4,96)=106.2$; $p<0.0001$) and a significant treatment*ROI interaction ($F(24, 294)=1.71$; $p=0.02$), but no statistically significant main effect of treatment ($F(2, 28)=1.27$; $p=0.30$). *Post-hoc* testing on the ROI*treatment interaction revealed a statistically significant increase in [^{3}H]Ro15-4513 specific binding in the dCA1 of rats exposed to 0.5 mg/kg/day haloperidol, as compared to rats exposed to vehicle ($p<0.01$; $q<0.01$; Cohen's d=1.3) or 2 mg/kg/day haloperidol ($p<0.05$; $q<0.01$; Cohen's d=-1.2) (Table 1; Figure 2). There were no statistically significant differences between 2 mg/kg/day haloperidol exposed rats vs. vehicle ($p=0.94$; $q>0.05$). In addition, in the NAc, exposure to 2mg/kg/day haloperidol decreased [^{3}H]Ro15-4513 binding relative to 0.5mg/kg/day haloperidol-exposed rats ($p<0.001$; $q<0.001$), although this did not reach statistical significance with respect to vehicle controls (vehicle vs 0.5mg/kg/day: $p=0.07$; $q=0.049$; vehicle vs. 2mg/kg/day: $p=0.06$, $q=0.049$) (Table 1; Figure 2). All other ROIs showed no statistically significant changes in [^{3}H]Ro15-4513 binding after 28 days exposure to haloperidol, irrespective of the dose.

[^{3}H]RO15-4513 binding

ROI	Vehicle	HAL 0.5mg/kg/day	HAL 2mg/kg/day	Veh vs. HAL0.5mg/kg/day	Veh vs. HAL 2mg/kg/day	HAL0.5mg/kg/day vs. HAL2mg/kg/day
Upper layer mPFC	2.78 (1.28, 8)	2.52 (1.06, 11)	1.97 (1.26, 9)	*p=0.64; q=0.68*	*p=0.21; q=0.50*	*p=0.32; q=0.50*
Deeper layer mPFC	5.99 (2.06, 9)	5.69 (1.38, 12)	5.22 (1.34, 10)	*p=0.71; q=0.75*	*p=0.35; q=0.66*	*p=0.42; q=0.66*
Upper layer ACC	2.37 (1.02, 8)	2.31 (0.73, 11)	1.76 (1.08, 8)	*p=0.90; q=0.94*	*p=0.27; q=0.42*	*p=0.24; q=0.42*
Deeper layer ACC	4.85 (1.31, 9)	4.66 (1.07, 12)	4.61 (1.03, 10)	*p=0.72; q=0.96*	*p=0.66, q=0.96*	*p=0.92; q=0.96*
CPU	0.44 (0.34, 5)	0.46 (0.40, 8)	0.48 (0.39, 4)	*p=0.94; q=0.99*	*p=0.90; q=0.99*	*p=0.95; q=0.99*
NAc	1.54 (0.77, 7)	2.30 (0.76, 9)	0.80 (0.53, 7) ##	*p=0.07; q=0.049*	*p=0.06; q=0.049*	*p<0.001; q<0.001*
dCA1	6.62 (1.05, 9)	7.99 (1.09, 12) *	6.66 (1.19, 10) #	*p<0.01; q<0.01*	*p=0.94; q=0.33*	*p<0.05; q<0.01*
dCA2	4.45 (1.52, 9)	4.56 (0.96, 12)	3.72 (1.36, 10)	*p=0.86; q=0.90*	*p=0.29; q=0.45*	*p=0.12; q=0.38*
DG	3.26 (1.19, 9)	3.44 (0.86, 12)	3.13 (0.95, 10)	*p=0.71; q=0.84*	*p=0.80; q=0.84*	*p=0.45; q=0.84*
dCA3	2.44 (0.83, 9)	3.01 (0.85, 12)	2.49 (0.86, 10)	*p=0.14; q=0.27*	*p=0.90; q=0.94*	*p=0.17; q=0.27*
vCA1	4.85 (2.16, 3)	3.71 (1.20, 12)	2.94 (0.83, 9)	*p=0.46; q=0.48*	*p=0.26; q=0.41*	*p=0.10; q=0.31*
vCA3	3.68 (3.12, 4)	3.35 (1.65, 12)	3.98 (1.02, 9)	*p=0.85; q=0.90*	*p=0.86; q=0.90*	*p=0.29; q=0.90*
Amy	2.11 (0.71, 9)	1.99 (0.68, 12)	1.83 (0.79, 10)	*p=0.72; q=0.76*	*p=0.44; q=0.76*	*p=0.61; q=0.76*

Table 1. Regional binding (nCi/mg) of [^{3}H]RO15-4513 across the ROIs explored, data show mean (SD, N). Prefrontal Cortex (PFC), Anterior Cingulate Cortex (ACC), upper layer (1-3) and deeper layer (4-6); Caudate-Putamen (CPu), Nucleus Accumbens (Nac); dorsal hippocampal layers CA1 (dCA1), CA2 (dCA2), CA3 (dCA3), dentate gyrus (DG); ventral hippocampal layers CA1 (vCA1), CA3 (vCA3); amygdala (Amy). *Vehicle vs. Haloperidol 0.5 mg/kg/day (q<0.01), # Haloperidol 0.5 mg/kg/day vs. Haloperidol 2 mg/kg/day (q<0.01), ## (q<0.001)

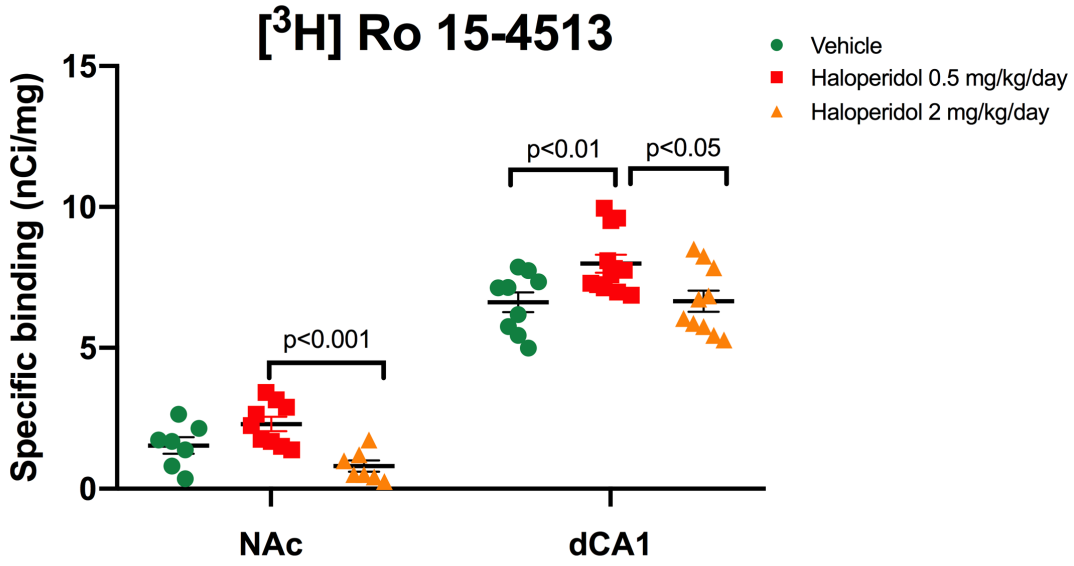

Figure 2. Chronic haloperidol exposure results in dose-specific changes in [^{3}H]RO15-4513 specific binding relative to vehicle controls in the nucleus accumbens (Nac) and dorsal Cornu Ammonis 1 (dCA1). Data points represent the specific binding values per individual animal (nCi/mg), horizontal line indicates group mean, bars indicate SEM.

General increase in [^{3}H]flumazenil binding after haloperidol exposure measured with quantitative autoradiography

Mixed-effects model ANOVA revealed a significant main effect of ROI (F=(4, 114)=124.2; p<0.0001) (Table S4, Figure S2) and treatment (F(2, 28)=3.86; p=0.03), but no ROI*treatment interaction (F (24, 304)=1.22; p=0.23). Across ROIs, the effect of haloperidol exposure was generally to increase [^{3}H]flumazenil-specific binding (see Table 2 and Figure 3).

[^{3}H]Flumazenil binding

ROI	Vehicle	HAL 0.5mg/kg/day	HAL 2mg/kg/day
Upper layer mPFC	28.22 (8.2, 7)	35.43 (9.15, 11)	34.58 (7.95, 9)
Deeper layer mPFC	33.81 (7.91, 9)	40.62 (8.18, 11)	40.93 (8.37, 9)
Upper layer ACC	35.41 (3.04, 8)	39.04 (8.84, 12)	40.89 (8.39, 9)
Deeper layer ACC	27.05 (6.48, 9)	35.95 (7.52, 12)	35.56 (8.83, 9)
CPU	5.81 (2.83, 9)	8.62 (3.79, 12)	6.86 (2.73, 8)
NAc	10.75 (4.33, 9)	15.43 (5.83, 12)	18.99 (5.58, 9)
dCA1	30.87 (3.36, 8)	29.03 (3.89, 10)	34.54 (4.17, 10)
dCA2	15.89 (5.37, 9)	16.89 (5.27, 11)	19.54 (4.07, 10)
DG	26.57 (5.52, 9)	29.00 (5.84, 11)	32.58 (4.24, 10)
dCA3	17.25 (5.39, 9)	18.55 (5.36, 11)	22.10 (4.21, 10)
vCA1	14.81 (4.48, 5)	19.93 (7.07, 11)	21.81 (4.11, 9)
vCA3	14.24 (8.03, 5)	18.43 (7.53, 11)	24.24 (5.29, 9)
Amy	27.31 (8.11, 8)	31.08 (6.61, 11)	34.68 (8.48, 10)

Table 2. Regional binding (nCi/mg) of [^{3}H]flumazenil across the ROIs explored, data show mean (SD, N). Prefrontal Cortex (PFC), Anterior Cingulate Cortex (ACC), upper layer (1-3) and deeper layer (4-6); Caudate-Putamen (Cpu), Nucleus Accumbens (Nac); dorsal hippocampal layers CA1 (dCA1), CA2 (dCA2),CA3 (dCA3), dentate gyrus (DG); ventral hippocampal layers CA1 (vCA1), CA3 (vCA3); amygdala (Amy).

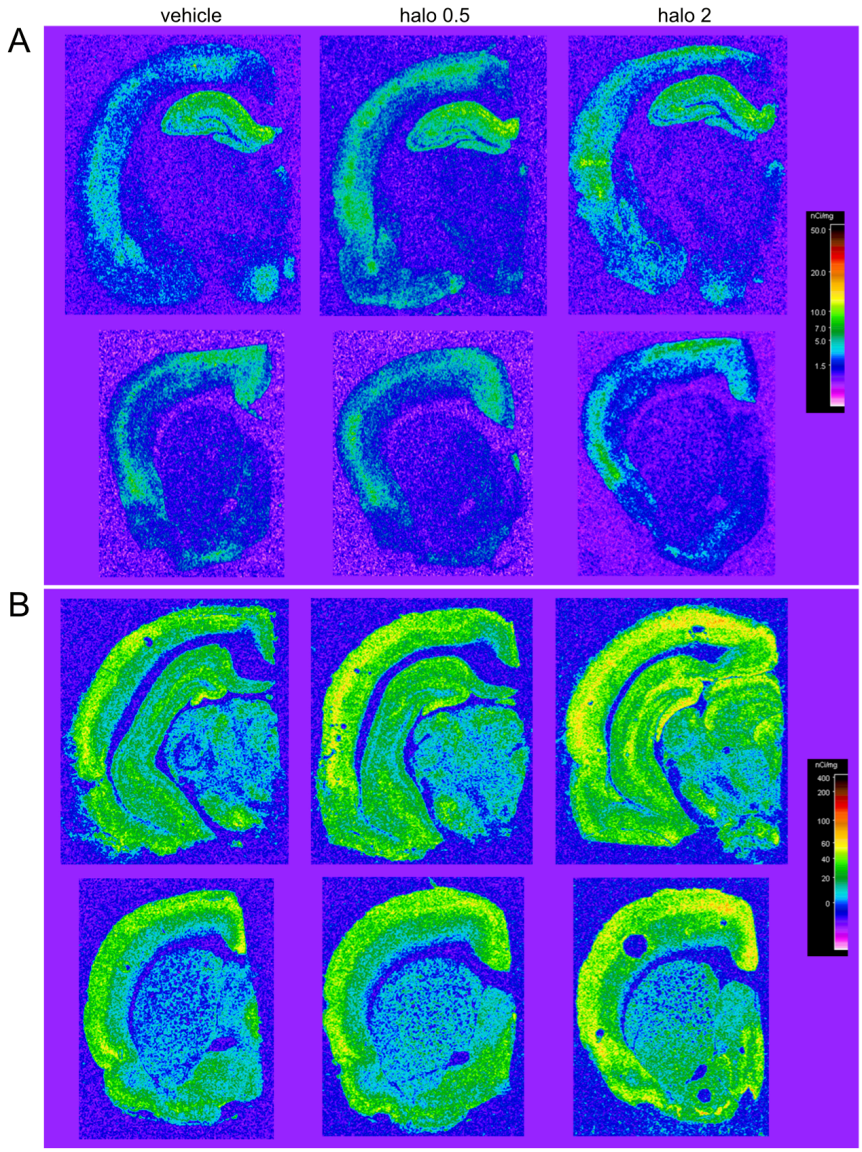

Figure 3. Pseudocolored representative autoradiographs showing A) [^{3}H]Ro15-4513 binding patterns and B) [^{3}H]flumazenil binding, by group: vehicle, haloperidol 0.5 mg/kg/day (halo 0.5), and haloperidol 2 mg/kg/day (halo 2).

Discussion

To our knowledge, this is the first study to investigate the effects of chronic exposure to haloperidol using clinically comparable dosing on $GABA_AR$ binding in a receptor subtype-specific manner using quantitative autoradiography. The main findings are that chronic haloperidol exposure results in a dose-specific increase in [^{3}H]Ro15-4513 binding in the dCA1 of the hippocampus. Precisely, [^{3}H]Ro15-4513 binding is increased

in rats exposed to haloperidol 0.5 mg/kg/day compared to rats exposed to vehicle with no effect of 2 mg/kg/day haloperidol. In contrast, chronic exposure to haloperidol generally increased [^{3}H]flumazenil binding in most ROIs irrespective of the dose administered. We found no relationship between these changes in ligand binding and vacuous chewing movements, a proxy measure for haloperidol-induced tardive dyskinesia, or drug plasma levels. These data suggest that chronic exposure to haloperidol has distinct, dose- and region-specific effects on the availability of $GABA_AR$ with specific sub-unit compositions. The present findings confirm that the dose and duration of exposure to haloperidol (and perhaps other antipsychotics) should be considered when measuring and interpreting $GABA_AR$ binding availability from schizophrenia patients.

Previous autoradiography studies have reported mixed findings regarding the effects of haloperidol exposure on $GABA_A$-BZR binding sites. McLeod and Colleagues (2008) reported an overall decrease in [^{3}H]flumazenil binding across cortical and subcortical regions after a 12-day exposure to 1 mg/kg of haloperidol administered once daily via an intraperitoneal injection [34]. In contrast, using a more clinically relevant mode of administration and longer duration of exposure (28 vs. 12 days), our data suggest chronic haloperidol exposure results in a generalized increase in [^{3}H]flumazenil binding across several brain ROIs. The present findings are in concordance with previous research suggesting that chronic haloperidol exposure is associated with increased $GABA_A$-BZR density in cortical areas using [^{3}H]flunitrazepam [32,33]. The discrepancies between our findings and those in McLeod and colleagues (2008) could simply reflect methodological differences, due to the differing modes of drug administration [34]. Notably, intraperitoneal administration of haloperidol does not result in clinic-like steady-state plasma levels or pharmacokinetics [37]. Alternatively, there could be bi-phasic, time-dependent effects of haloperidol exposure on $GABA_AR$ availability. McLeod and

colleagues (2008) also reported no effect of 12-day haloperidol exposure on zolpidem-insensitive [^{3}H]flumazenil binding, which is suggested to reflect α5GABA$_A$ binding sites [34,38]. Hence, it may be speculated that [^{3}H]Ro15-4513 binding might be affected differently following shorter or longer exposure to haloperidol which should be explored by studying clinically-relevant dosages of antipsychotic medication at different time points.

The use of quantitative autoradiography in the present study provides proof-of-concept evidence that exposure to haloperidol affects GABA$_A$-BZR as well as α5GABA$_A$R specifically. This technique in pre-clinical research is highly advantageous as it provides better spatial resolution than PET studies and sets a translational framework for conducting clinical research with homologue ligands, which other techniques such as histology do not allow. Collectively, our findings and those of others suggest that haloperidol, a D2R antagonist, likely impacts on GABA neurotransmission within the hippocampal-midbrain loop circuitry, which is critically involved in emotion salience and memory and is dysregulated in schizophrenia [61]. Precisely how these drug-induced changes in GABA$_A$R binding relate to central GABA neurotransmission however remains unclear. Quantitative autoradiography reflects changes in binding of the ligands to available receptor sites, which may relate to either upregulation or downregulation of neurotransmitter release. For example, an increase in receptor binding could indicate a compensatory effect in the form of a reduction in neurotransmitter levels. An increase in GABA levels does nonetheless also enhance affinity of GABA$_A$R for BZ-ligands such as flumazenil via a conformational change [62–64]. This is not the case, for BZR inverse agonists such as Ro15-4513, in which increased GABA levels appear to decrease the affinity of GABA$_A$R for this ligand [65]. Hence, increases in [^{3}H]flumazenil binding

could reflect either increased or decreased GABA levels, whilst decreases in [^{3}H]Ro15-4513 binding likely reflect increases in GABA levels and vice versa. Taken together, our observations of increased [^{3}H]Ro15-4513 binding in the dCA1 following exposure to 0.5 mg/kg/day haloperidol may be interpreted as evidence for increases in membrane α1/5-$GABA_AR$ in response to decreased GABA levels [60]. In the higher-dose haloperidol group, we observed vacuous chewing movements, which in rats, it is an analogue measure to tardive dyskinesia observed in humans and can be an indicator of almost complete D2R blocking occupancy [66], that may be causing differential effects on α1/5$GABA_AR$ than the lower-exposure dosages.

The overall increased [^{3}H]flumazenil binding could be suggestive of increased GABA release. Notably, *in vitro* slice electrophysiology suggests that D2R mediate GABA release onto pyramidal neurons in the PFC, whereby GABA release is decreased following dopamine administration [67]. D2R antagonists, such as haloperidol, would be predicted to increase GABA levels in the rodent frontal cortex, which could lead to elevated [^{3}H]flumazenil binding. In support of this view, GABA-immunoreactivity is increased in the axosomatic terminals of neurons in layers II, III, V, and VI in the frontal cortex of rats exposed chronically to 0.5 mg/kg/day haloperidol over 4 months [68]. In contrast, a microdialysis study in rats reported a decrease of extracellular levels of GABA in the nucleus accumbens region following chronic haloperidol exposure [61], suggesting a compensatory upregulation of $GABA_AR$ in our sample of rats.

Studies of bulk tissue GABA levels in the frontal cortex of schizophrenia patients using proton magnetic resonance spectroscopy (^{1}H-MRS), however, report either no effect [70,71] or a normalisation of elevated GABA levels [54] following antipsychotic

exposure. The precise nature of the relationship between $GABA_AR$ binding and GABA levels following antipsychotic exposure therefore remains to be confirmed in future studies using corroborative methods, including microdialysis, ^{1}H-MRS and immunohistochemistry to map the cellular localization of these receptor changes. Of particular interest would be further exploration of the subiculum and dCA1 areas of the hippocampus, based on the present findings and on their involvement in the pathophysiology of psychosis [56,61,72].

Limitations of our study should be noted. First, while [^{3}H]Ro15-4513 binds predominantly to diazepam-sensitive $GABA_A$ sites, it also binds to a diazepam-insensitive site in the cortex and hippocampus with lower affinity [73], which should be taken into consideration when comparing [^{3}H]Ro15-4513's binding patterns to those of the BZ-sensitive ligand [^{3}H]flumazenil. Second, much of the binding was present within the lower-range of the commercially available standards, which limited the ability to detect a specific signal from regions with lower-binding values (likely reflecting lower receptor density). Hence, we may have underestimated or missed effects of haloperidol in such regions. Third, we only examined the effects of haloperidol; therefore, it is unclear whether the findings reported would generalize to other antipsychotic drugs such as olanzapine, aripiprazole and clozapine. In our previous studies concerning effects of haloperidol and olanzapine on brain volume and cellular markers, we found no clear differences between these compounds [74,75]. Whilst we have no reason to believe that olanzapine for example, would not induce similar effects to haloperidol, this should be explicitly tested in future studies. Future studies should also address sex as a biological variable, since we only used male animals. Finally, it should be taken into consideration that the present data were collected in naïve animals, which do not recapitulate any

features relevant to the pathophysiology of schizophrenia. Hence, haloperidol may act differentially on the GABA system in an initially dysregulated, diseased system. Future studies should therefore investigate the effects of antipsychotics on $GABA_AR$ in animal models reflective of genetic, environmental or pharmacological risk factors for psychosis.

In summary, our findings indicate that chronic treatment with haloperidol induces dose-specific changes in α1/5$GABA_AR$ in the dCA1 and a generalized increase in BZ-$GABA_AR$ in healthy rodents. These findings suggest that the mechanisms of action of antipsychotics may also involve the modulation of $GABA_AR$ in the midbrain-hippocampal loop, predominantly implicating α1-3;5$GABA_AR$. These mechanisms may be led more specifically by α5 receptors, particularly in the dCA1 and in a dose-specific manner. The present results align with the notion that administration of antipsychotics can change the responsivity to novel GABA-targeting drugs [76]. Importantly, the ligands used in this study to explore α5$GABA_AR$ (Ro15-4513) as well as BZ-sensitive $GABA_AR$ (flumazenil) offer promising approaches for translational research as they can be used in cross-species studies including PET in humans [77].

Funding and disclosure

This work was partly supported by a Sir Henry Dale Fellowship jointly funded by the Wellcome Trust and the Royal Society to GM (#202397/Z/16/Z). ACV acknowledges the financial support of the Medical Research Council (New Investigator Research Grant MR/N025377/1 and Centre Grant MR/N026063/1). ACV discloses receipt of research funding from F. Hoffman La Roche Ltd and UBC Biopharma SPRL as part of a research program on early life immune activation. The views expressed are those of the authors and not necessarily those of F. Hoffman La Roche or UCB Biopharma SPRL. These funders had no influence on the decision to publish this work.

Author contributions

DC, NS, AV and GM designed the experiments. MCC conducted the surgeries and tissue preparation. NS, DC, AK, APY conducted the autoradiography experiments. APY and DC analyzed the data. All authors contributed to writing the manuscript.

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